ADDIS ABABA UNIVERSITY

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Evaluation of isolates of *Pseudomonas fluorescens* as biocontrol agent against potato bacterial wilt caused by *Ralstonia (Pseudomonas) solanacearum*

By

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ABSTRACT

A total of 50 bacterial isolates of fluorescent pseudomonas were isolated from potato rhizosphere soil collected from different potato growing areas of the country. The isolates were screened *In Vitro* for their antagonistic activity against the pathogen, *Ralstonia solanacearum* on KB agar medium. Out of the 50 isolates, only three (Pfs2, Pfw1, Pfwt3) showed a certain degree of inhibition to the growth of the pathogen. To test their antagonistic effect in a greenhouse, a pot experiment was conducted using sterilized soil and potato tuber (CIP 383032.15) that was susceptible to the pathogen. In the experiment, bacterization of potato tubers with the selected isolates *i.e.*, Pfs 2, Pfw 1, Pfwt 3 and Pfri (reference strain) significantly reduced the incidence of bacterial wilt by 59.83%, and increased plant growth: plant height and dry weight by 76.89% and 28.44%, respectively suggesting the importance of the isolates as plant growth promoting rhizobacteria.

Key words/Phrases: Potato*, P. fluorescens, R. solanacearum,* Antagonstic Interaction

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ABBREIVATION

APPRC: Ambo Plant Protection Research Center

ATP: Adenosine tri phosphate

CIP: International Potato Center

DNA: Deoxy ribonucleic acid

DRB: Deleterious Rhizobacteria

DRMO: Deleterious rhizosphere microorganism

EARO: Ethiopian Agricultural Research Organization

EDDA: Ethylene diaminedi (o-hydroxyphenyl) acetic acid

EDTA: Ethylene diamine tetra acetate

Fe-EDTA: Ethylene diamine tetraaceto ferrate

FP: Fluorescent pseudomonas

HARC: Holleta Agricultural Research Center

HCN: Hydrogen cyanide

ISR: Induced Systemic Resistance

KB: King's B medium

LPS: Lipo polysaccharide

NADH: Nicotinamide adenine dinucleotide

PGPR: Plant Growth Promoting Rhizobacteria

SAR: Systemic Acquired Resistance

SPL: Scientific Phytopathological Laboratory

SPSS: Statistical Package for Social Science

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DECLARATION

I, the undersigned, declare that this thesis is my original work and all sources of material used for this study have been dully acknowledged.

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wilt disease is one of the main factors that limit potato production in the country (Berga Lemaga, 1986).

Bacterial wilt also known as brown rot is caused by *Ralstonia (Pseudomonas) solanacearum E.F Smith,* a soil-borne bacterium. It is one of the most destructive and bacterial plant diseases that are predominantly distributed in the tropical, subtropical and warm temperate regions of the world. It affects a wide range of plants as many as 200 plant species representing more than 50 families of particularly members of solanaeceous such as potato, tomato, etc (Hayward, 1995). It is responsible for yield loss of potato to the extent of 50-80% in Kenya, Burundi and Uganda (Ajanga, 1993; Skoglund, *et al.*, 1993) and also 70% in India (Sinha, 1986).

In Ethiopia, the presence of bacterial wilt was first reported on potato and eggplant in Keffa region (Stewart, 1956). Later, the occurrence of the disease on potato, tomato and eggplant was reported in Showa and Arsi region too (Stewart and Dagnachew, 1967). Although systematic loss assessment studies have not been done in Ethiopia, a preliminary survey conducted on, wilt incidence of potato showed a percentage increase to the tune of 0.8-21%, 3.8-24% and 1.5-45% at Tsedey, Bako and Ziway, respectively (SPL, 1986). Studies regarding the diversity of the pathogen showed that the strains that occur in Ethiopia belong to race 3 of biovar 2 of *R .solanacearum* (Yaynu Hiskias, 1989; Ketema Abebe, 1999).

The current, incomplete understanding of intraspecific diversity coupled with gaps in knowledge on dissemination, infection and disease development have hampered the development of effective disease management strategies. As a result, no universal control measures exist which are effective across the wide host range of the pathogen (Cook *et al*., 1989).

The common control measures employed against bacterial wilt include the use of resistant variety, crop sanitation, crop rotation, selection of disease free planting material and other cultural practices. Control through the use of resistant varieties, has showed little success. This is because such kind of resistance is strain specific, liable to break down at an ambient temperature by virulent and highly polymorphic strains of *R .solanacearum* and in nematode infested soil (French and Lindo, 1982; Prior *et al*., 1994). Successful control of the pathogen through crop rotation is also not always effective since rotation practices recommended for one area may not perform well at other locations and also differences in the strains involved (Prior *et al*., 1994).

Biological control of diseases caused by soil-borne plant pathogens, by the use of antagonist microorganism has been the focus of study for more than 70 years (Baker, 1987). Recently, interest in this area has been increased because of concern over the potential hazard of chemical pesticides to the environment and public health, declining efficacy of pesticides due to the development of resistance by the pathogen and absence of effective chemical control. Therefore, biological

control has become the possible alternative to chemical pesticides and many successful examples of disease control such as bacterial wilt have been reported (Mazzola, 1998).

The use of rhizosphere resident microbial antagonist specially the fluorescent pseudomonas is noted as a promising control method (Cronin *et al*., 1999). The term"rhizosphere" was introduced by Hiltner in 1904, and is defined as a volume of soil surrounding plant roots in which plant growth is stimulated. The rhizosphere is a habitat in which several biologically important processes and interactions takes place (Sorensen, 1997). This rhizosphere effect is primarily due to the influx of mineral nutrients to plant roots through mass flow and diffusion, along side the efflux and accumulation of plant root exudates, a complex mixture of chemicals and organic compound secreted into the soil by the root drive the underground interactions (Bias, 2004).

The rhizosphere is characterized by a complex and dynamic microorganisms that are classified into plant growth promoting rhizobacteria (PGPR) and plant growth inhibiting rhizobcateria (PGIR). The PGPR are antagonistic to a pathogen and provide first line of defense for the plant against pathogen (Mazzola, 1998). Furthermore, they enhance plant growth and yield by fixing atmospheric nitrogen, solubilizing minerals (such as phosphorus), producing plant growth regulators (hormones) and producing siderophore that sequester iron (Glick, 1995).

Fluorescent pseudomonas are often selected for biological control because of their ability to utilize varied substrates under different conditions, short generation time and motility that assist colonization of roots. Moreover, they produce variety of antagonistic secondary metabolites such as antibiotics, siderophore, etc, responsible for the biological suppression of several soil borne plant pathogens (Bagnasco *et al*., 1998).

Sunaina *et al*., 1997 reported that fluorescent pseudomonas strains when applied to potato seed pieces were found to reduce the population of *Eriwinia cartovora* on roots and tubers by 95-100% and 28-95 %, respectively. In related study, Gamliel and Katan (1993) found that inoculation of Fluorescent pseudomonas decreased the incidence disease caused by *Sclerotium rolfsii* in bean, *Fusarium* wilt in cotton and tomato.

Similarly, Schroth and Hankock (1981) reported that application of fluorescent pseudomonas increased yield of potato and root weight of radish by 5-33%, 60-144%, respectively. In another study, Aspiras and Cruz (1986) treated *R .solanacearum* infested soil with *B. polymixa* and *P*. *fluorescens* that result in increased survival of tomato to 60% and 90%, respectively. Therefore, this study was initiated with the aim to isolate and characterize different isolates of *P. fluorescens* from the rhizosphere of potato soil. And to screen and evaluate its antagonistic effect against *R. solanacearum* in relation to the growth performance of potato plant.

2. Literature Review

2.1 The Genus Pseudomonas

The genus Pseudomonas is gram negative, non-sporeforming, and straight or slightly curved rod shaped bacteria. They are strictly aerobic, chemoorganotrophic, motile by polar flagella, oxidase and catalse positive having the GC content of 58-71 mol% (Palleroni *et al.,* 1986).

It is the most diverse and ecologically significant group of bacteria found in large numbers in all natural environments and also forms intimate associations with plant and animals. This universal distribution reflected in a remarkable degree of physiological and genetic diversity (Spiers *et al*., 2000).

Members of the genus are endowed with wide metabolic capability, which enables them to degrade a variety of compounds that are highly refractory to other organisms, including aliphatic and aromatic hydrocarbons, fatty acids insecticides and other environmental pollutant (Spiers *et al*., 2000). This is because the genus is rich in regulatory genes that regulate the spatial and temporal expression of genes, a crucial factor affecting the ability of these bacteria to take advantage of novel ecological and genetic opportunity presented by gene transfer events. It also posses large genomic size which are highly dynamic accumulating ecologically useful sequences by gene transfer and losing gene by deletion if they no longer provide useful function in a broad range of ecological niches (Lawerence, 1999).

2.2 Taxonomy and Classification of *R.solanacaerum*

The genus *Ralstonia* consist the phytopathogenic bacteria. Previously, it was with in the genus Pseudomonas and later it has been reclassified into the current genus as a result of molecular investigation (Taghavi *et al*., 1996).

The pathogen comprises of both the virulent and avirulent (mutant) type. The avirulent mutant, form a small butyrous colonies with a distinct dark red color on 2,3,5 triphenylterazoliumchloride agar medium (TZC) while the virulent wild type form irregularly-round, fludial, white colonies with light pink center producing massive extracelluar polysaccharides (Hayward, 1964).

R .solanacearum is a complex species containing strains differing in a host range, geographical distribution, physiological and biochemical characteristics, pathogencity and epidemiological relationships (Buddenhagen *et al*., 1964).

Two principal approaches that have gained acceptance in the classification of *R. solanacearum* are race and biovar types suggested by Buddenhagen and Kelman (1964) and Hayward (1964), respectively. Race and biovar are informal groupings at the infra sub specific level that are not governed by the code of nomenclature of bacteria.

The race classification is based on host range and geographical distribution of strains (Buddenhagen and Kelman, 1964; He *et al*., 1983). Previously, three races were identified. Race 1 is composed of strains that are widely distributed

throughout the warmer region of the world affecting a wide range of solaneceous and other plants, mainly potato, tomato, eggplant, chill, peanut and several weeds.

Race 2 consists of strains primarily affecting musaceous hosts such as plantin, banana and *Heliconia spp*. It may also affect potatoes and occasionally tomato, but not other crops. Race 2 is indigenous to America and its native host is *Heliconia*, but the plantin and banana introduced from Asia and Africa provided the selection pressure for new strains to emerge from the indigenous population (French, 1986).

Race 3 has a narrow host range being restricted to potato, tomato and few weeds in nature. It primarily affects potato and hence sometimes called potato race occurring in the cool climates. Lately, two additional groups of strains have been reported, one from Philippines affecting ginger (Zehr, 1970) and the other from China affecting mulberry (He *et al.,* 1983). The two groups were suggested to be race 4 and 5, respectively.

The biovar classification is based on the physiological properties of strains. Thus, the strains have been classified into five biovars according to their ability to oxidize three disaccharides namely, lactose, maltose and cellobiose and three hexose alcohols, manitol, sorbitol, and dulcitol (Hayward, 1964; He *et al.,* 1983).

Biovar 1 consists of strains that oxidize neither group of carbohydrates. Strains that are capable of oxidizing the disaccharides except the hexose alcohol are designated as bovar 2. Strains assigned in biovar 3 oxidize both carbohydrates.

Strains capable of oxidizing the hexose alcohol but not the disaccharides are grouped as biovar 4.The strains from mulberry are found to be distinct in that they oxidize the three disaccharides and manitol but not dulcitol and sorbitol and hence designated as biovar 5 (He *et al.,* 1983). Thus, biovar 1 and 2 are less nutritionally versatile than biovars 3, 4 and 5.

Therefore, there are marked differences in the geographical distribution of biovars suggestive of separate evolutionary origin. Since this classification depends on different character, a race may not fully correlate to a specific biovar except that race 3 is equivalent to biovar 2 (Buddenhagen, 1986). Even in this exception the reverse may not be necessarily true because there are biovar 2 strains that are not race 3.In this regard table 1 depicts the classification and distribution of *R .solanacearum* in Ethiopia.

Table 1. Geographical distribution and classification of *R .solanacearum* in Ethiopia

Administrative Region	District/major Location	Classification		Host plant	Species	RFLP division
		Biovar	Race			
East Showa	Shashamane	2	3	Potato	Solanceaous	Group II
West Showa	Jeldu	2	3	Potato	Solanceaous	Group II
	Inchini	2	3	Potato	Solanceaous	Group II
	Ambo	2	3	Potato	Solanceaous	Group II
	Bako	2	3	Potato	Solanceaous	Group II
Sidama	Wondogenet	2	3	Potato	Solanceaous	Group II

Source: Yaynu Hiskias (1989) and Ketema Abebe (1999)

For better understanding and characterization of *R .solanaecarum,* the relationships of all strains of the biovar and races to each other and other related species were studied and the phylogentic relationships were evaluated using restriction fragment length polymorphism (RFLP) (Cook and Sequeira, 1993). The RFLP pattern analyses revealed that strain of the species have common DNA fragment with two types of sequence orientation.

Accordingly, all race 1 biovars 3, 4,and 5, designated as Division I were very much a like with more than 78% sequence similarity. Race 1 biovar 1 and biovar 2 and 3 strains designated as Division II were also found to be alike with more than 62% similarity.However, the similarity between the two divisions was found to be 13.5 %(Cook and Sequeira 1989). Thus, it was suggested that the two groups can be regarded as subspecies.

The studies also showed that *R .solanaecarum* is distantly related to other species of pseudomonas and the only species that showed relationships with *R.solanacearum* were *P.picketti, and P.yzygi* (Roberts *et al.,* 1990). Thus it was concluded that *R .solanacearum* strains are homogeneous and distinct group that bears little relationships to other plant pathogenic pseudomonades.

2.3 Pathogenesis of *R.solanacearum* in the Host

Plant pathogens have evolved different strategies to invade their host. One of such strategies uses specialized cell structure for penetration of epidermal cell like, haustoria in fungi, stylet in nematodes, etc (Agrios.1978). Since bacteria lack such kind of structures it is incapable of mechanical penetration in the cutinized plant tissues. Generally, they use natural opening such as stomata, hydathodes and lenticels as a route of entrance and also through wounds caused by insects, nematodes, farm tools and other microorganisms (Rangaswami and Rajagopalan, 1973).

The main route of infection of *R .solanacearum* is usually through the wounded part of the vascular tissue *i.e*., xylem. In the wounded region only a few bacteria

are needed to cause infection and the incidence and severity of bacterial wilt disease increase in area where the population of root rot nematode is high (Kelman and Sequeira, 1965).

Active invasion of healthy root occur via the lateral root emergence points is possible bacterial cells (Kelman and Sequeira, 1965). This is because the pathogen is endowed with an enzyme that enable them to digest their way through the mucilagenous coating of the emerging root which are pectinous in nature. That is, *R .solanacearum* produce enzymes such as endonglucanases (cellulase) and polygalactouranse (pectinase) that can degrade cellulose and pectin, respectively (Allen *et al*., 1993; Denny *et al*., 1993) which also serve as an important virulence factor during pathogensis. Inaddition the gene, *pehA*, controlling pectinase synthesis were identified. Therefore the pathogen can penetrate and get in to the host through the healthy part of the root by using these enzymes. Then the bacteria get in to the xylem tissue digesting the primary wall of the weakened cortical cell using cellulase.

Following entry in to the xylem vessel, *R .solanacerum* multiplies free of competition. And to overcome condition that can inhibit its development, the bacterium has developed three strategies. The first one is avoidance of hypersensitive response induction, which is rapid defense mechanism that restricts further spread of the invading pathogen in non-host plant. (Arlat *et al*., 1993). While the second strategy aim at prevention of its attachment to cell wall,

particularly when multiplying in intracellular spaces by the use of bacterial extracellular polysaccharides (EPS). The last strategy involves facilitation of nutritional status of the xylem fluid through production of growth regulators. This stimulates xylem parenchyma to redifferncniate and divide, so that the nutrients are re-routed in to the infected xylem. This enables the bacterium to obtain sufficient sugars and amino acids (Sequeira, 1993).

Generally, wilt symptoms are produced by various bacterial action on the plant .In this case the bacterium produce a slimy substance (extra cellular high molecular weight polysaccharide) surrounding the bacterial mass, this increases the viscosity of vessel fluids and movement of water declines and finally resulting wilt. Thus, the extracellular polysaccharides play a vital role in the process of wilt induction. Inaddition, pectic and cellulytic enzymes also participate to the wilting process too (Kelman and Sequeira 1965).

Eventually, the bacteria using its enzymes get in to the host move laterally, form cavities and then released into the environment upon the collapse of the tissue through the points of the emergence of the lateral roots and the disease cycle continues (Sequeira, 1993). Generally, the disease is characterized by a rapid wilt having a short incubation period, mostly with in 48 hours in succulent annuals. In hardy perennials it may take longer, a month or more (Rangaswami and Rajagopalan, 1973). However, symptom development may also depend on the strain type, host susceptibility and environmental factors.

2.4 Taxonomy and Classification of *P. fluorescens*

The genus Pseudomonas was subdivided in to five rRNA homology groups (rRNA group I to V), based on the percentage similarities of the various Pseudomonas species by rRNA:DNA hybridization. New molecular analyses and taxonomic rearrangement identified the rRNA group I species as members of a phylogenetically homogenous group referred to as *Pseudomonas* (Palleroni *et al*., 1973). This classification agrees with phylogenetic information obtained from 16S rRNA sequence data.

The fluorescent pseudomonas (*P.fluorescens,P.putida*) are known to improve plant growth and implicated in the biological suppression of various soil borne diseases and also in the biodegradation of natural or man made toxic chemical compounds (Holloway,1992).

Pseudomonas fluorescens is one of the most ubiquitous and diverse species this led to the subdivision of the species into five biovars (Johnson and Palleroni, 1989). This diversity is associated with their wide metabolic capability and a variety of habitats including soil, water, plants, animals and their products and human clinical specimens. Woese (1987) reported that environmental and trophic factors play a major role in the evolution and speciation of these ubiquitous bacteria. The subdivision of *Pseudomonas fluorescens* into their respective five biovars is based on their biochemical characteristics that were proposed by Palleroni (1992) and Stainer *et al* (1966). However, this method of characterization

has two limitations, (I) It attributes too much importance to some properties and (II) also takes small number of properties into account.

In order to avoid this limitation, numerical taxonomy is employed which consider a large number of phenotypic properties, where all properties are given the same importance and analyzed mathematically in order to classify to strain level (Sneath *et al*., 1981). Here, biovars in *P.fluorescens* do not exactly match the delineated phenotypic cluster: some strains belonging to different biovars are classified into the same cluster and other strain from the same biovar is classified into different phenons. For this reason genotypic characterization of the species and biovars is required to classify the real taxonomic status of the subdisvion.

Wayne *et al.,* (1987) defined species as cluster of bacterial strains showing a level of similarity of their DNA greater than 70% by DNA-DNA hybridization with difference in the denaturation of temperature of their DNA less than 5^0C.Palleroni *et al* (1973) studied the relationships between biovars of *P.fluorescens* using DNA-DNA hybridization technique and reported that the presence of large genomic variability among them. The hybridization percentage between the biovars were found to be lower than 70%, which indicated the presence of high diversity with in the biovars of *Pseudomonas fluorescens,* this agreed with, Palleroni (1992) report that is *P.fluorescens* along with *P.putida* comprise up to 500 species.

Similarly, Lagurre *et al* (1994) applied restriction fragment length polymorphism (RFLP) analysis in the taxonomy of fluorescent pseudomonas. And its result correlate well with the DNA-DNA hybridization of Palleroni *et al* (1973) confirming the heterogeneity of strains belonging to *Pseudomonas fluorescens*.

2.5 Siderophores of Pseudomonas fluorescens

Siderophores (Gr. Iron bearers) are low molecular weight (500-1000 daltons), virtually Fe (III) specific ligand produced by microorganisms under iron stress designed to supply iron to the microorganism (Neilands, 1981). It is a molecule with a bidenate ligand, which usually with oxygen atoms, contacts Fe (III) with its six-octahedric bonds.

Fluorescent pseudomonas produce yellow-green, water soluble, siderophore called pyoverdin or pseudobactin. The pyoverdins comprise family of structurally related compounds that differ in amino acids composition and configuration .The chromophore component that is identical for all pyoverdin imparts the color and fluorescence and its catechol unit is one of the binding sites for iron (III) (Meyer, 2000).

Since ferric-siderophore complex are usually too large to diffuse through the small water filled porin channel in the cell envelope of the gram-negative bacteria, active iron transport is initiated by outer membrane receptor proteins for ferric-sideroprore (Neilands, 1982). The synthesis of this receptor protein is also induced

by iron limiting condition. Inner membrane and periplasmic proteins are also required for ferric siderophore transport (Neilands, 1982).

Release of iron from the ferric siderophore complex proceeds via reduction since siderophores have little affinity for Iron (II). The released iron is then incorporated into iron containing cellular constituents where it may interact as iron (II) with repressor protein to regulate the biosynthesis of the siderophore and receptor protein for the ferric siderophore (Neilands, 1981).

2.5.1 Siderophore and Plant Growth Stimulation

Many strains of fluorescent pseudomonas are known to enhance plant growth or yield and suppress disease when applied as seed inoculants (Elad and Chet, 1987) are called Plant Growth Promoting Rhizobacteria (PGPR). Seed bacterization with fluorescent pseudomonas for disease suppression, plant growth and yield increament is becoming an emerging field in plant biotechnology.

The enhanced plant growth is ascribed to the production of siderophores by fluorescent pseudomonas under iron limiting condition, which starves the deleterious rhizosphere microorganisms (DRMO) of their ferric iron (Fe^{+3}). The pathogens owe their deleterious activity through production of hydrogen cyanide (HCN) in the presence of Fe^{+3}, which adversely affect the energy metabolism of the root cells (Baker and Schippers, 1987).

Uptake of nutrients by plant roots is an energy requiring process. Respiratory energy used for nutrient uptake is of considerable importance and amounts to 60% of the root respiration under normal condition. (Walker *et al*., 2003) The production of ATP, mediated by cytochrome oxidase respiration, can be inhibited by cyanide because its production depends on Fe (III) availability. This inhibition causes electrons released by oxidation of NADH in potato mitochondria to follow the alternative cyanide - resistant respiratory path way to oxygen where much energy is lost as heat instead of being used for phosphorylation of ADP (Baker and Schippers, 1987).

Kloepper *et al.*, (1980) reported that amending the field soil with iron (III) in the form of ethylene diamine tetra aceto ferrate (III) (Fe- EDTA) avoid the plant growth enhancement characteristics that would occur due to application of the beneficial strains in green house experiment and it also abolishes the *In Vitro* bacterostatic activity against pathogen.

This is confirmed by the use of an isolated yellow green, fluorescent siderophore called pseudobactin, from iron limiting culture of *Pseudomonas fluorescens* strain B10. Pseudobactin exhibited bacteriostatic activity against the pathogen where as the red -brown ferric pseudobactin displayed no activity. This result suggests that the *In Vitro* antibiosis of beneficial strain (*P.fluorescens)* against the pathogen is caused in part by iron deprivation induced by the siderophore of the beneficial strain (Kloepper *et al.,* 1980).

In green house assay the application of both Pseudomonas fluorescent strain B10 as a cell suspension and pure pseudobactin at 10μM caused significant increases in plant growth compared with water treated control. Neither ferric pseudobactin at 50μM nor *strain B10* with 50μM Fe-EDTA increased plant growth (Kloepper *et al*., 1980).

Furthermore, mutants obtained by exposure to ultraviolet light, mutagenic chemicals or by transposon mutagenesis, induce not only loss of the ability to produce siderophore *In Vitro*, but also their plant growth stimulating properties, although they colonized roots equally well like the wild one (Leong, 1986).

Therefore, root-colonizing beneficial fluorescent pseudomonas enhance plant growth in part by producing siderophore, which efficiently sequester iron (III) and making it unavailable to the pathogen that will be utilized in HCN production.

2.5.2 Environmental Factors Affecting Microbial Iron Availability

The synthesis and secretion of siderophore molecules by the bacterial cell is controlled by Fe^{+3} concentration, temperature, pH, soil texture, rate and composition of exudates by the plant root, etc. Only at Fe^{+3} concentrations lower than approximately 20μM does the cell switch on its genetic capacity for the biosynthesis and secretion of the siderophore and for the biosynthesis of the outer membrane receptor protein (Meyer, 2000).

The availability of Fe (III) is limited in alkaline and neutral soil, and increases with increasing soil acidity. Therefore, high deleterious activity or less suppression by

Fluorescent pseudomonas is expected with increasing soil acidity. The frequency and relative proportion of certain clay minerals seems to have a pronounced effect on sideroprhore mediated iron transport. For example, the presence or absence of particular clay minerals is correlated with the suppressiveness of certain soil to soil borne fungal and bacterial disease (Leong, 1986).

2.6 Root Colonization

The success of plant growth promotion by the introduction of *Pseudomonas fluorescens* depends largely on their timely establishment and persistence throughout the growing season at sites where DRMO may become active (Baker and Schippers, 1987). Introduction of fluorescent pseudomonas through seeds or tubers, from which they will colonize the developing root system have been detected in the rhizosphere of potato plant throughout the growing season, although their relative numbers gradually decline in the later season (Bakker *et al*, 1986).

In accordance with this Schipper *et al*., (1987) reported the presence of tuber yield difference between early and late harvesting that result high and low yield, respectively. This difference might be explained by the reduction of relative numbers of introduced Pseudomonas later in the season and also change in the root-exudate composition of the aging potato plant where the amount of proline, which is the precursor of HCN, increased dramatically that favor the DRMO under this condition.

A successful fluorescent pseudomonas after introduction becomes distributed along the root in natural soil, propagates and survives for several weeks in the presence of competition from the indigenous rhizosphere micro flora (Weger *et al*., 1998)

2.6.1 The Process of Root Colonization

The process of root colonization of an introduced strain of fluorescent pseudomonas has two phases (Howie *et al.*, 1987). Phase I begins as introduced bacteria on seeds or seed pieces come into contact with the emerging roots. Some kind of attachment of the cells to the root surface may be essential for initiation of phase I. As the root elongates, some of the bacteria are carried along with the root tip, while others are left behind as a source of inoculum on older portion of the root. Bacterial multiplication at the tip would permit transport of bacteria as long as the roots grow, but without multiplication, transport would occur only until the initial inoculum at the root tip is diluted out (Howie *et al.*, 1987).

The ultimate fate of an introduced bacterium is governed to a large extent by its ability to compete with the indigenous micro flora during phase II. Rhizosphere competent bacteria will multiply and survive on the root; where as incompetent bacteria are rapidly displaced. Dupler and Baker (1984) reported that *P.putida* N - 1R colonized the radish rhizosphere less efficiently when the bacteria were added to biologically active soil than when the same soil was air dried prior to the test to reduce microbiological activity, competition would have been greater in the former soil.

Nutrients rather than space are thought to be the limiting factor in competition among bacteria during rhizosphere colonization. Therefore, bacteria tend to congregate in grooves between cells where nutrient may be most abundant. Since the carrying capacity of the rhizosphere is limited, an introduced strain pre-empt the establishment of indigenous bacteria if it is to become established. Thus, in response to an introduced strain, the total population of rhizosphere microorganisms may not change, but rather the composition of the population is altered (Weger *et al*., 1998).

2.6.2 Factors Affecting Root Colonization

The distribution of introduced bacteria along the root during phase I, and their propagation and survival during phase II, are profoundly affected by biotic and abiotic factors. Howie *et al* (1987) reported high bacterial growth on roots with in a range of 0.3 to 0.7 bar matric potential in which oxygen, nutrient availably and turgor potential of the cell was optimal for the growth of *P. fluorescens 2-79*.

The movement of bacteria through soil by means of water is affected by bacterial characteristics such as cell shape, size, buoyancy, motility and electrostatic charge. Channels left by old roots or worms and possible gaps at the root soil interface created by the diurnal shrinking and swelling of roots might allow more rapid downward movement of bacteria in water than occurs along roots in a more uniform soil matrix (Parke *et al.*, 1986).

The optimal temperature for growth of *P .fluorescens and P. putida invivo* is 25-30^{0}C but root colonization by these bacteria is generally greatest below 20^{0}C. Microbial activity in the soil increases as soil temperature increase; thus better colonization at lower temperature probably reflects less competition from indigenous micro flora (Loper *et al,* 1984).

Plant genotype influence the quantity and composition of the rhizosphere micro flora (Glick, 1995) possibly through differences in root exudates, and manipulating host genotype may offer some opportunity to improve the efficiency or consistency of root colonization by introduced bacteria.

2.7 Mechanisms of Pathogen Suppression

2.7.1 Competition and Niche Exclusion

Competition is one of the mechanisms of disease control by biological means where the DRMO and the PGPR compete for nutrient and suitable niches on the root surface. Competition for nutrients supplied by root exudates occurs between antagonistic bacteria and pathogens on the root are responsible for the biocontrol activity by introduced bacteria. Large populations of bacteria established on planting material and roots could act as sink for nutrients in the rhizosphere, thus reducing the amount of nutritional availability for the pathogen stimulation and subsequent colonization of the root (Elad and Chet, 1987). This is mainly due to their nutritional versatility and high growth rate of the fluorescent pseudomonas in the rhizosphere.

In the same way Suslow (1982) suggested that the niche exclusion is potentially an important mechanism of antagonism of pathogen by fluorescent pseudomonas. Certain areas on the root such as cell junctions and points of emergence of lateral roots appear to be favored for colonization by many kinds of bacteria including the pathogen because root exudates are abundant there. Therefore, inoculating-planting material with fluorescent pseudomonas presumably prevents or reduces the establishment of the pathogen at these sites.

2.7.2 Siderophore Mediated Disease Control

The ability to produce specific siderophore and /or to utilize a broad spectrum of siderophore plays a vital role in the use of fluorescent Pseudomonas in the biological control of phytopathogen. This is because the produced siderophore scavenge the ferric iron in the rhizosphere and ultimately suppresses the growth of the pathogen (Leong, 1986).

Kloepper *et al* (1980) reported the possibility of changing a pathogen conducive-soil to a pathogen suppressive one by adding to the conducive soil either a *Pseudomonas strain B10*, isolated from soil suppressive to ¨take-all¨ or its siderophore. Flax seeds which were treated with suspension of *strain B10* (10^9cfu/ml) and planted in conducive soil infested with *F.oxysporium f.sp.lini*, increased survival of seedlings from 48% to 87% when compared to non-inoculated seedlings. Similarly, the suppressiveness of a soil to ¨take-all¨ or fusarium wilt was eliminated when Fe-EDTA at 50µM was added to the soil. In this case, less than 47% flax seedlings survived in soil infested with *F.oxysporium*

f.sp.lini and amended with Fe-EDTA than in the same soil infested but not amended with Fe-EDTA. The number of flax seedlings that survived in non infested soil with and with out added Fe-EDTA were the same as the number of surviving seedlings in infested soil with out added Fe-EDTA. This suggests that fluorescent pseudomonas present in the soil were producing siderophoore, which sequester iron (III) and make it unavailable to the pathogen (Kloepper *et al.,* 1980).

The synthetic Fe (III) chelating agent ethylene diamine di(o-hydroxyphenyl) acetic acid, EDDA, and its iron complex, Fe-EDDA also induced suppressiveness to ¨take-all¨. The iron from Fe-EDDA is used by the plant, leaving the soil iron-free, EDDA, which induce suppressiveness by depriving the pathogen iron(III).The ability of synthetic chelator ,EDDA,to mimic the biological action of yellow –green, fluorescent siderophore by this strain under iron limiting condition helps to account for pathogen suppression with population of specific fluorescent pseudomonas in soil (Wong and Baker,1984).

The availability of iron (III) in the soil declines with increasing soil P^H. Thus siderophore-mediated suppression is greater in neutral and alkaline soils than in acid soil (Bakker *et al,* 1986). Pathogens are thought to be sensitive to suppression by siderophore for several reasons (a) they produce no siderophore of their own (b) they are unable to use siderophore produced by the antagonists or by other microorganisms in their immediate environment (c) they produce too little siderophore or a siderophore with a little affinity for iron than those of antagonist;

or (d) they produce a siderophore that can be used by the antagonist, but they are unable to use the antagonists siderophore (Hankock, 1981;Leong, 1986).

2.7.3 Induced Systemic Resistance

Induced protection in plants against various pathogens by biotic and abiotic inducers have been reported since 1930s.Induced systemic resistance (ISR) is a rhizobacterially mediated systemic résistance that do not cause any damage to the plant, which is in contrast to systemic acquired resistance (SAR) where resistance is induced by pathogen and results in activation of resistance mechanism in uninfected parts that inhibit or resist further spread (Chen *et al.*, 2000).

The inducing bacteria are mostly saprophytic and can simultaneously induce resistance and plant growth. Transposon mutagenesis experiment has indicated factors involved in induced resistance are lipo polysaccharide (LPS) O-antigenic carbohydrate side chain and the siderophore of fluorescent pseudomonas (Leeman *et al*., 1995). A bacterial component or its metabolite is perceived by the plant root through binding to a receptor to an intracellular signal. There after, the metabolite itself, or signal generated by the plant cell initiates a cascade of signal transduction. Eventually, the translocated signal is perceived by distant plant cells, triggering the activation of the defense arsenal of the challenged host plant (Leeman *et al*., 1995).

Induced systemic resistance once expressed activates multiple defense mechanism that include: strengthening of epidermal and cortical cell walls and

deposition of newly formed barriers beyond infection site including callose, lignin and phenolics; increased levels of enzymes such as chitinase, peroxidase; enhanced phytoalexin production; enhanced expression of stress related genes (Benhamou *et al*., 2000).

The most interesting aspect of induced systemic resistance is its ability of controlling wide spectrum of pathogens with a single inducing agent. Therefore, application of fluorescent pseudomonas mediated induced systemic resistance holds promise for practical disease management.

2.7.4 Antagonism by Antibiosis

One of the mechanisms by which biocontrol and plant growth promotion occurs in the rhizosphere is by the production of antibiotics. It is an organic substance that is produced by microbes and deleterious to the growth or metabolic activities of other microorganism (Fravel, 1988).

Fluorescent pseudomonas are found to produce a variety of antibiotics that confer an organism a selective advantage in competition for nutrients and space with in their ecological niches. It helps the bacteria to colonize root surface and improve their ecological fitness that leads to disease reduction and plant growth (Fravel, 1988).

The role of antibiotics in biocontrol has been determined by generation of mutant that donot produce antibiotics. *Pseudomonas fluorescens* is suppressive to a

number of phytopathogenic bacteria through production of a phenazine antibiotic (Thomashow and Weller, 1987). But *Tn5* mutagenesis makes the mutants defective in the production of phenazine antibiotic. Then all the mutant become non inhibitory to the growth of the pathogen both *In Vitro* and *In vivo*. Biocontrol ability was restored in the mutant strain when antibiotic production was restored by wild type DNA introduced as cosmoid library (Thomashow, *et al*., 1986).

3. MATERIAL AND METHODS

3.1 Study Areas

The study areas selected for this research work are known for their agricultural productivity. The areas receive good amount rainfall and have diverse climatic condition, which favor the cultivation of different agricultural crops. Potato is one of the horticultural crops, produced in these areas both for local consumption as well as export.

A. Ambo

Ambo district is located in the West Showa at 8^{O}57'N latitude,37^{O}52' E longitude and 2200m(a.s.l) which is about 125km away from Addis Ababa. The potato growing area has sandy loam, which is favorable for potato production. The major challenge for potato production in this area are late blight and bacterial wilt, which are caused by *Phytophthora infestans* and *Ralstonia solanacearum* respectively.

B. Bako

Bako is also found in the West Showa, which is situated 250km from Addis Ababa. It has an annual rainfall of 1395.18mm with the mean daily minimum and maximum temperature of 14.67 and 28.53^{O}C, respectively. Maize is the dominant crop in production when compared to other cereal and legume crops in the area. Among the variety of horticultural crops produced, potato is important. The major constraint of potato production in this area is bacterial wilt that occurred since 1979 (SPL, 1980) causing damage to potato and tomato.

C. Shashamane

Shashamane is located East Showa zone at 7^{O} 13`N latitude and 38^{O}35'E longitude 250km away from Addis Ababa. The area is known for the production of different cereal, legume and horticultural crops. Potato is the leading horticultural cash crop to local farmers. Bacterial wilt is one of the major limiting factors of potato production in this area, which cause huge amount of yield loss annually.

D. Wondogenet

Wondogenet is found in the, Southern Showa that is 267km from Addis Ababa. Since the area is rich in water resources potato is produced during the dry season under irrigation and in the rainy season too. Bacterial wilt imposes huge problem for potato production in this area throughout the year.

E. Wolayta

Wolayta is situated in the Southern region of the country that is 300km from Addis Ababa. The area is one of the most populated areas in the region and it is well known for its potato production, as main food and income source to the local farmers.

3.2 Soil sampling

Soil samples were collected from healthy, good performing as well as diseased potato plant randomly. After collection each soil sample were kept separately and then transported to the National Soil Research Center, Addis Ababa for isolation of the bacterial isolates.

3.3 Tobacco Hypersensitivity Reaction (HR)

Since there is a possibility that the pathogenic bacteria may lose it's virulence and become a virulent upon storage, a preliminary pathogencity and hypersensitivity test was done to confirm its virulence and race 3 strain of *R .solanacearum*.

The pathogenic isolate Rs 262-b obtained from Ambo Plant Protection Research Center was tested for virulence on tobacco leaf following the method of Lazano and Sequeria (1970). In brief: bacterial suspension of the pathogen was prepared from 48-72 hours old culture at a concentration of 10^9 cfu/ml (Klement *et al.*, 1964). Then the suspension was infiltrated to the lower surface of a tobacco leaf cv white Burley by a using syringe. Control plants were infiltrated with sterile distilled water. Following inoculation plants were kept at room temperature and reactions were observed and recorded daily.

3.4 Pathogencity Test

Potato tubers of the susceptible cultivar (CIP 383032.15) obtained from Holleta Agricultural Research Center (HARC), Potato Research Division were planted in 20cm diameter plastic pots (one plant per pot) filled with sterilized soil.

At three leaf stage potato plants were injected with bacterial suspension containing 10^9 cfu/ml prepared from 48-72 hour old culture, by stem puncture method of Winsted and Kelman (1952). Suspension of the isolate was inoculated into the stems of the test plant at the axile of the second or third leaf from the apex. Control plants were injected with sterile distilled water. After inoculation the plants were put in an incubator at a temperature of 28^0C for 48 hours and then transferred to green

house where the minimum and maximum temperature was 14.8^0c and 35.2^0c respectively.

3.5 Isolation of Fluorescent pseudomonas

Soil samples were collected from potato growing areas of the country during the period October-November, 2003. Then the bacteria were isolated following the method of Vlassak, *et al.*, (1992) 1gm of each the soil sample was shaken in 100 ml of phosphate buffered solution (PBS) solution (0.88 %(w/v) NaCl, 2.9mM KH_2PO_4, 7.1 mM$KHPO_4$, P^H 7.2) for 2 hours on a rotary shaker at 200 rpm. PBS diluted extracts were plated on modified king's B (KB) medium containing 100 mg cyclohexamide, 12.5mg chloroamphenicol and 50mg ampicilin. After incubation at 28^0c for 24 hr representative types of colonies were further purified on KB agar medium and pure isolates were preserved on KB slant and stored at 4^OC.

3.6 Characterization of Isolates

Morphological features and color of colonies of the isolates were determined using King's B agar medium composed of proteose petone (20gm), glycerol (15ml),K_2HPO_4(1.5gm),$MgSO_4.7H_2O$ and agar (20gm) in a litter of distilled water(King *et al.*,1954).

Gram reaction of the isolates was determined by using potassium hydroxide solubility test, according to Suslow *et al.*, (1982). A single colony was taken and mixed with a drop of 3% potassium hydroxide (KOH) solution with a loop on glass slide until an even suspension is obtained. The formation of mucous thread upon

lifting with the loop was taken as an indication of the presence of the gram-negative bacteria.

Catalase activity was determined by the addition of a drop of 3% hydrogen peroxide (H_2O_2) to a 24-hour-old colony and production of gas bubbles was taken as a positive result (Goszczynska *et al.*, 2000).

Oxidase test was conducted according to Goszczynska *et al.,* (2000). Twenty-four hour old bacterial colonies were used for the test, where one loopful of bacterial culture grown on nutrient agar medium was transferred to Whatman No. 1 filter paper moistened with a solution of 1%N, N, N, N-tetraethyl P-Phenyldiaminedihydrochloride. Appearance of intense purple color instantly with in 10 seconds was taken as positive.

Starch hydrolysis was determined by cultivating the isolates on nutrient agar supplemented with 0.2% starch. After 2-5 days, cultures were flooded with Lugol's Iodide solution. Appearance of yellowish, clear zone around or under bacterial growth was taken as an indication hydrolysis of starch. (Goszczynska *et al.,* 2000).

For determination of *levan formation*, isolates were spread on nutrient agar medium supplemented with 5% sucrose (Goszczynska *et al.,* 2000), incubated for 3 to 5 days.The observation of white, domed and mucoid, shining slimy colonies was taken as an indication of levan formation.

The ability of bacterial isolates to grow at high or low temperature was determined on KB plate. Plates that were spot inoculated were incubated at $4^{O}C$ and $41^{O}C$, and the presence or absence of growth was monitored for 10 days.

Detection of the *production of fluorescein* by the isolates was conducted on KB medium. Twenty-four hour old culture of the isolates were streaked on the plate and incubated at a temperature of $28^{O}C$, after 24-48 hours colonies were examined for fluorescens under a source UV light according to King *et al*., (1954).

Gelatin liquefaction test was conducted on gelatin agar composed of agar (15gm), Gelatin (15gm), peptone (4gm), yeast extract (1gm) with a P^{H} 7.2 autoclaved for 15 min at 15 pascal. Twenty-four hour old culture of the isolate was streaked on the plates and incubated at a temperature of $28^{0}C$. Hydrolysis of gelatin was then detected by a transparent zone surrounding spot-inoculated growth upon addition of acidified mercuric chloride (6gm of $HgCl_2$, 8ml of concentrated hydrochloric acid, 40 ml distilled water) according to Pickett *et al*.,(1991).

Salt tolerance of the isolates was determined on KB plate containing 1,2,5%(w/v) NaCl following the method described by Goszczynska *et al.,* (2000).

Siderophore production of catechol type was detected by Arnow test (1937). 1ml of cell suspension prepared from 24hour old culture was taken and to which 1ml of 0.5N hydrochloric acid was added followed by the addition of 1ml nitrite-molybdate reagent (10 gm sodium-nitrite and 10gm sodium-molybdate dissolved in 100ml

water) and 1ml 1N sodium hydroxide. The production of red color which was stable for 1hour taken as an indication of the presence of catechol type siderophore.

Carbohydrate utilization test was made on standard basal medium with test carbohydrates. The medium contains g/L, $MgSO_4.7H_2O$ (0.2gm), KCl (0.2gm),$NH_4H_2PO_4$(1gm),Bromothymolblue (1ml),agar (12gm), and the P^H was adjusted to 7.2.The medium was heated to boiling until the ingredient were melted and dissolved and then sterilized at 121^OC for 15 minutes .The carbohydrates were prepared separately as 10% solutions and autoclaved except glucose, arabinose and galactose which were filter sterilized. Melted basal medium was dispensed in sterile flask in 90ml quantities. After cooling to 50^Oc, 10ml of each carbohydrate solution was added to make 1% and poured into Peteri dishes. Then, a single colony of 24-hour-old cultures of each isolate was inoculated into the medium with three replication. Uninoculated medium was used as control and all Peteri dishes were incubated at 28^OC and monitored for 14 days for any color change. A change to yellow color was taken as positive for the test.

3.7 *In vitro* Inhibition Test

All isolates were first screened for their toxicity toward the pathogen on king's B medium (KB) agar plates in dual culture assays (Ganesan and Gnanamanickam, 1987). KB plates were prepared by mixing suspension of cells scarped from 48-72 hours old culture of the pathogen with cooled and molten KB agar (42^0C). The agar suspension was then dispensed into Peteri dishes and allowed to solidify.

Sterile loop was used to spot inoculate the test strain from a 24 hour old culture of the isolates into the middle surface of the solidified bacterial lawn. (Skathivel and Gnanamanickam, 1987). Like wise, KB agar plate spot inoculated with water were used as a control. Assay plates were maintained at 28^{O}C and observed for inhibition zones after 2 to 3 days.

Bacterial designation

Pfs: refers to isolates of *Pseudomonas fluorescens* from Shashamane area

Pfwt: refers to isolates of *Pseudomonas fluorescens* from Wondogenet area

Pfw: refers to isolates of *Pseudomonas fluorescens* from Wolayta area

Pfb: refers to isolates of *Pseudomonas fluorescens* from Bako area

Pfa: refers to isolates of *Pseudomonas fluorescens* from Ambo area

Pfri: refers to reference strain of *Pseudomonas fluorescens* from India

Rs262-b: refers to *Ralstonia solanacearum* (pathogenic isolate) kindly provided by Ambo Plant Protection Research Center

3.8 Green House Experiment

The greenhouse experiment was conducted at the National Soil Research Center, in Addis Ababa with an altitude of 2394 m.a.s.l with minimum and maximum temperature of 19-25^{O}C and 25-31^{O}C,respectively.

Based on the *in vitro* antibiosis result, three isolates of *Pseudomonas fluorescens* were chosen for this experiment. The three selected isolates Pfwt 3, Pfw 1, Pfs 2 and Pfri (one reference strain introduced from India) were grown on KB agar medium for 24 hours and diluted to give a suspension which was adjusted optically

to concentration of 10^9 cfu/ml (OD_{600}=1.0) (Mulya *et al*., 1996). Potato tuber (CV CIP 383032.15) highly susceptible to bacterial wilt obtained from Holleta Agricultural Research Center, Potato Research Division were surface sterilized with disinfectants before treatment.

The soil was autoclaved at 121^{O}C for one hour and amended with fertilizer (N: P: K=2:1:1) (Mulya *et al*., 1996) at a rate of 10g per pot as a solution. Three kilogram sterilized soil were filled into the plastic pots with a diameter of 20cm, which were surface sterilized with alcohol into which two potato tubers were planted with the following treatment.

Treatment applied were :-

T1. Rs 262-b (pathogen inoculated)

T2. Pfw 1 treated (Wolayta isolate)

T3. Pfs 2 treated (Shashamane isolate)

T4. Pfwt 3 treated (Wondogenet isolate)

T5. Pfri treated (Indian reference strain)

T6. Pfw 1 treated and Rs 262-b inoculated

T7. Pfs 2 treated and Rs 262-b inoculated

T8. Pfwt 3 treated and Rs 262-b inoculated

T9. Pfri treated and Rs 262-b inoculated

T10. Control (Neither treated nor Inoculated)

Potato tuber in T1 was planted in soil infested with 100 ml of 10^9cfu/ml suspension of the pathogen (Sunaina *et al*., 1997), while in T2 to T5 each tuber was dipped in the suspension of each antagonist isolates for sixty minutes and planted in sterilized soil. But in T6 to T9 after dipping the potato tuber in each suspension for sixty minutes were planted in sterilized but pathogen inoculated (infested) soil. And potato tuber in T10 was used as control.

Plants were watered regularly with deionized water and treatments were arranged in a completely randomized design with three replication. Observation on percent survival and plant height was taken. Then, the plants were cut at the soil level and dry weights of the shoot were determined after oven drying at 60^OC for 72 hours.

3.9 Data Analysis

The experimental data was analyzed using one-way analysis of variance andcomparison of means at 5% level was made by Tukey's test. Statistical analysis was done using SPSS v12.0 (SPSS Inc., Chichago, IL., USA).

4. Results

4.1 Hypersensitivity Reaction

Injection of bacterial suspension of Rs 262-b isolate into tobacco leaves caused yellow necrosis with in 24 to 48 hours. Here, there was no any progress of necrosis to the adjoining tissues and wilting of the plant even after some weeks. In addition the control plant injected with distilled water remained healthy.

4.2 Pathogencity Test

The result of the pathogencity test showed that, the bacterial isolate was pathogenic to potato. Development of wilt on the potato plant was rapid and complete wilting of the plant occurred within 8 to 13 days. Infected potato plants exhibited epinasty, stunting and browning of stems upon dissection.

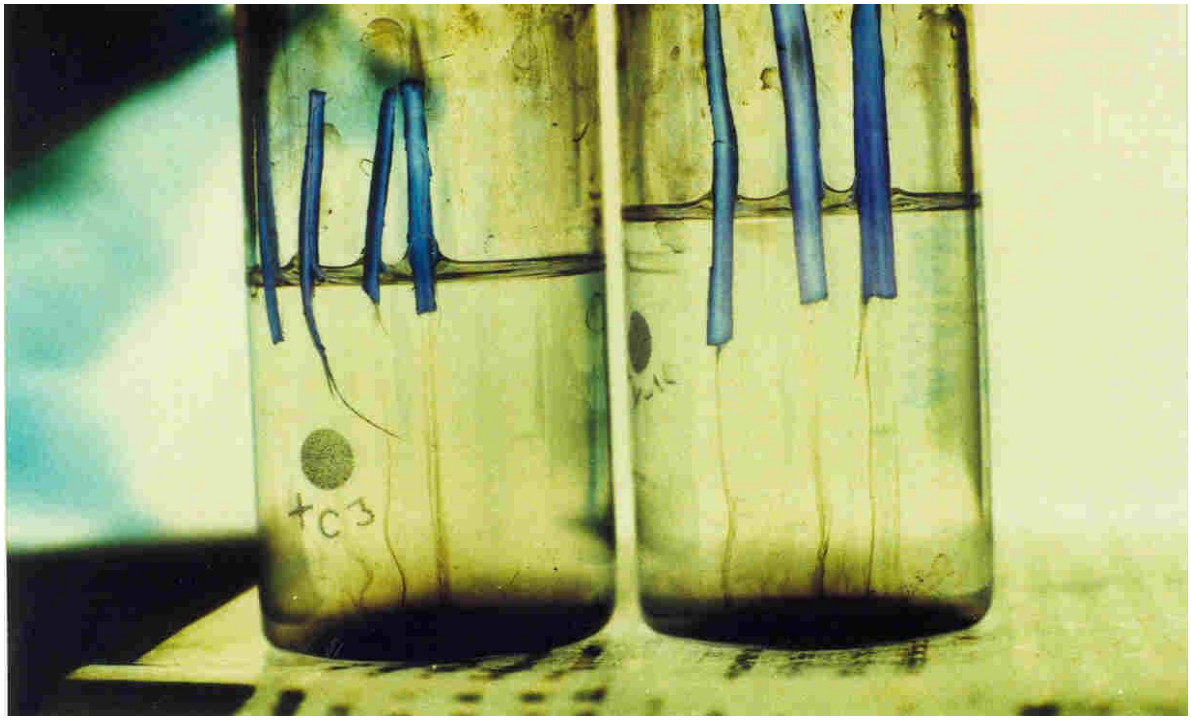

Figure 1. Bacterial streaming

4.3 Isolation and Characterization of Isolates

A total of 50 fluorescent bacteria isolates were isolated from soil samples collected from different potato growing regions of the country (Table 2).

All isolates produced yellow-green diffusible pigment of variable intensities on King's B medium. Morphologically, they were short rods and form levan on NA with 5% sucrose, which was white, doomed, and mucoid shining colony.

Table .2 Distribution of isolates of *P.fluorescens* in different potato growing areas and their cultural characteristics.

Origin	No.of isolates	Characteristics			
		Morphologgy	Colony apperance	Fluorescein production	Levan formation
Shashamanae	10	short rods	small shiny	+	+
Wondogenet	10	short rods	small shiny	+	+
Wolayta	15	short rods	small shiny	+	+
Ambo	8	short rods	small shiny	+	±
Bako	7	short rods	small shiny	+	±

Key: + =Positive reaction; **±**=Intermediate result

Biochemical properties of the isolates are presented in Table.4 All the isolates were found to be gram negative, oxidase and catalase positive. They were able to grow at $4^{O}C$ but not at $41^{O}C$ and tolerated NaCl concentration from 1 to 2% but not at 5%. They utilized the tested carbohydrates and produced yellow color on the medium, which was an indication of the utilization of each carbohydrate. In addition they liquefied gelatine but failed to hydrolyze starch. They also produced catechol type siderophore.

Table 3. Distinctive feature of *P.solanacearum* and *P.fluorescens*

Microorganism	Hypersensitivity reaction	Pathogencity test	Siderophore production	Antibiotic production
Ralstonia solanacearum	+	+	-	-
Pseudomonas fluorescens	ND	ND	+	+(-)

Key: **+** =Positive reaction; **-** =Negative reaction; **ND**=Not done

Table. 4 Biochemical characteristics of isolates of *P.fluorescens*

Characteristics	Names of isolates				
	Pfa	Pfb	Pfs	Pfwt	Pfw
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Siderophore detection	+	+	+	+	+
Growth on KB@ 41^{O}C	±	-	-	-	-
Growth on KB@ 4^{O}C	+	+	+	+	+
Salt tolerance @					
1%NaCl	+	+	+	+	+
2%NaCl	+	+	+	+	+
5%NaCl	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-
Gelatine liquefaction	±	±	+	+	+
Carbon source Utilization					
Arabinose	+	±	+	+	+
Galactose	+	+	+	±	+
Glucose	+	+	+	+	+
Fructose	±	+	+	+	+
Sucrose	+	+	+	+	+
Mannose	+	±	+	±	+

Key: **+** =positive for a reaction; **-** = negative for a reaction; **±**=Intermediate result

4.4 *In vitro* Antibiosis

Of the 50 fluorescent isolates screened against *P.solanacearum* on KB medium only three local isolates i.e., Pfwt 3 (Wondogenet), Pfw1 (Wolayta), Pfs1 (Shashamane) and Pfri (reference strain) were capable of inhibiting the growth of the pathogen while others failed to do so. The diameter of inhibition zones ranged from 1.2 to 2.4 mm where the minimum and maximum inhibition caused by *Pfri* and *Pfw1,* respectively table 6.

Table.5 *In vitro* antibiosis and zones of inhibition caused by the isolates

Origin	No.of isolates	Antbiosis	Name of isolates	Zone of inhibition (cm)
Shashamanae	10	1	PfS 2	1.5
Wondogenet	10	1	PfWt 3	1.32
Wolayta	15	1	PfW 1	2.4
Ambo	8	-	-	-
Bako	7	-	-	-
India	1	1	Pf ri	1.2

4.5 Green House Experiment

4.5.1 Bacterial Wilt Suppression by Bacteria Treatment

Data from the greenhouse experiment showed that only 40% of plant survived in T1 (where potato tuber planted in pathogen infested soil without bacterization). But potato treated with the selected antagonistic isolates prior to planting in pathogen infested soil significantly suppressed the incidence of bacterial wilt and also

increased its survival rate by 59.83% as compared to (T1). The control plant did not show any wilt symptoms and had 100% survival.

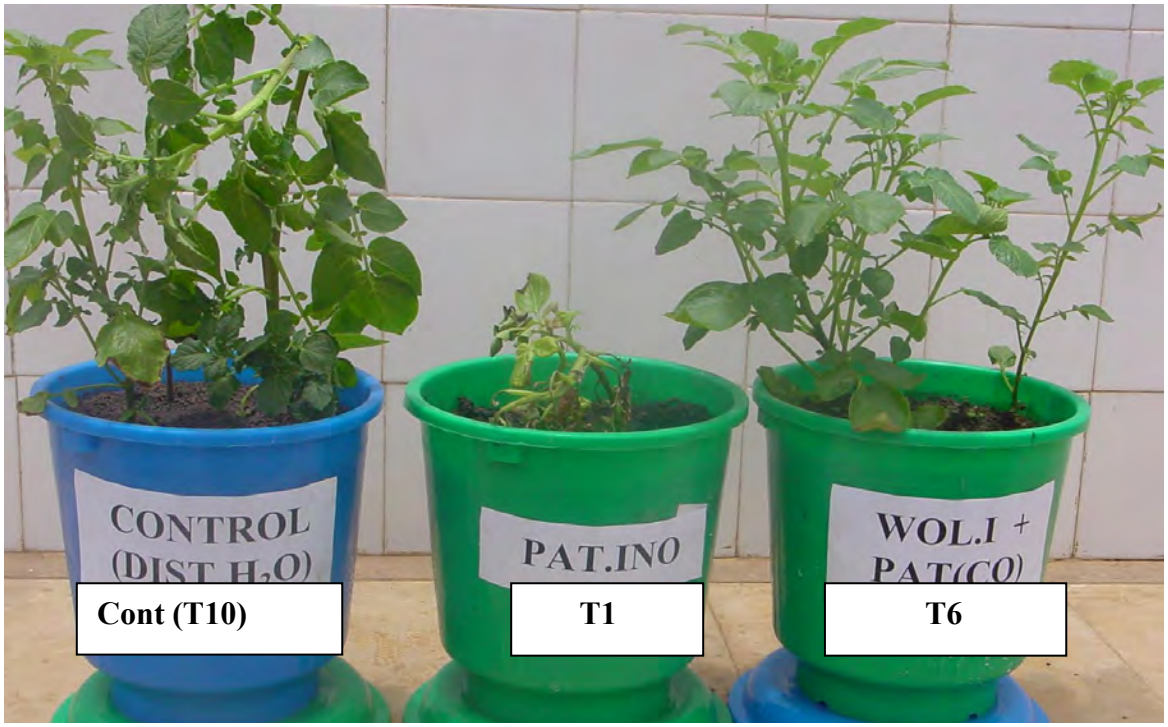

Figure 2. Greenhouse experiment showing difference between T1, T6 and T10

Table. 6 Suppression of bacterial wilt caused by the pathogen in plant treated with *Pseudomonas fluorescens*

Treatment	**Percent survival ·**
T1. Rs262-b ino.	$40.0^{b} \pm 2.34$
T2. Pfw 1 + Rs 262-b	$99.89^{a} \pm 0.85$
T3. Pfs 2 + Rs262-b	$99.8^{a} \pm 0.84$
T4. Pfwt 3 + Rs262-b	$99.83^{a} \pm 1.09$
T5. Pfri + Rs262-b	$99.8^{a} \pm 1.31$
T6. Control	$100^{a} \pm 1.00$

· Numbers are mean and SD of three replicate (two plant in each pot)

*Means followed by the same letter with in a column are not significantly different at α=0.05 by Tukey's test.

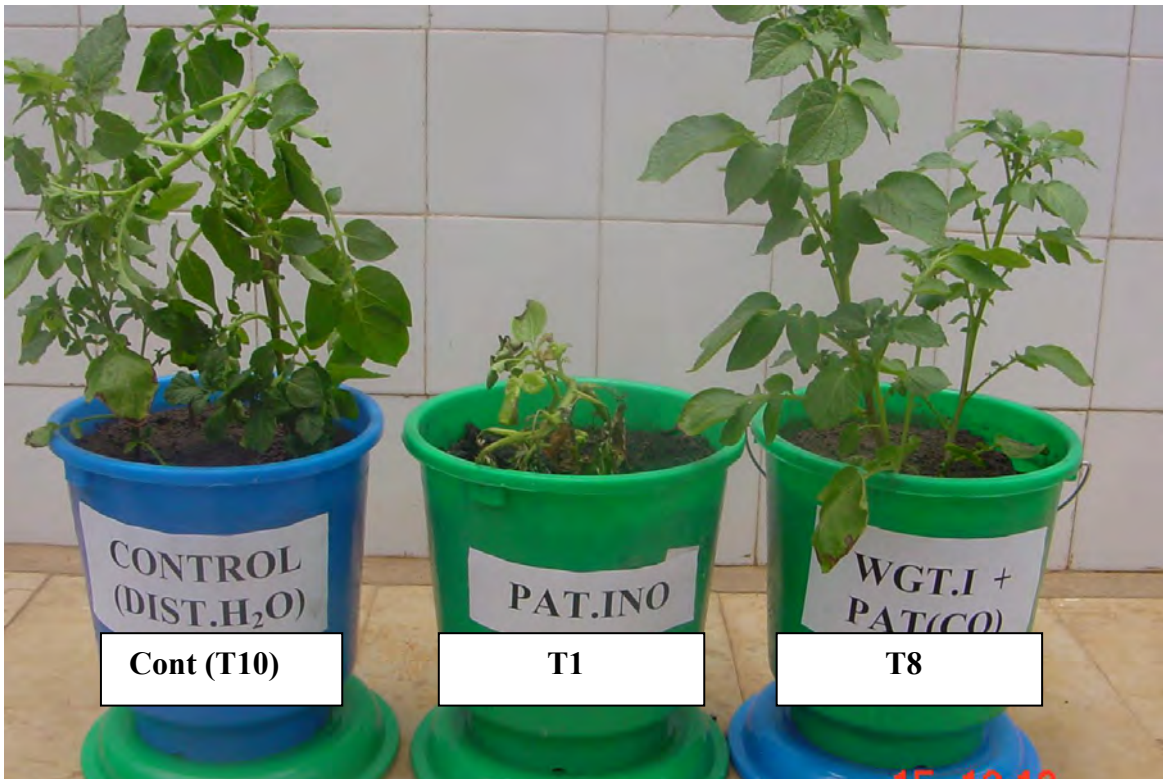

Figure 3. Greenhouse experiment showing difference between T1, T8 and T10

4.5.2 Effect of Bacteria Treatment on Plant Height and Biomass

Data on plant height and dry weight of potato are recorded in the Table 7.Plant height and biomass were significantly lower in the potato planted in pathogen-infested soil (T1) than the rest of all treatment (T2 to T10). Potato tuber treated with the selected isolates prior planting in pathogen free (T2-T5) and infested soil increased plant height and dry weight when compared to T1. However, *P.fluorescens* treated + pathogen inoculated treatment (T6-T9) didn't show an increased plant height and biomass better than the control (T10).

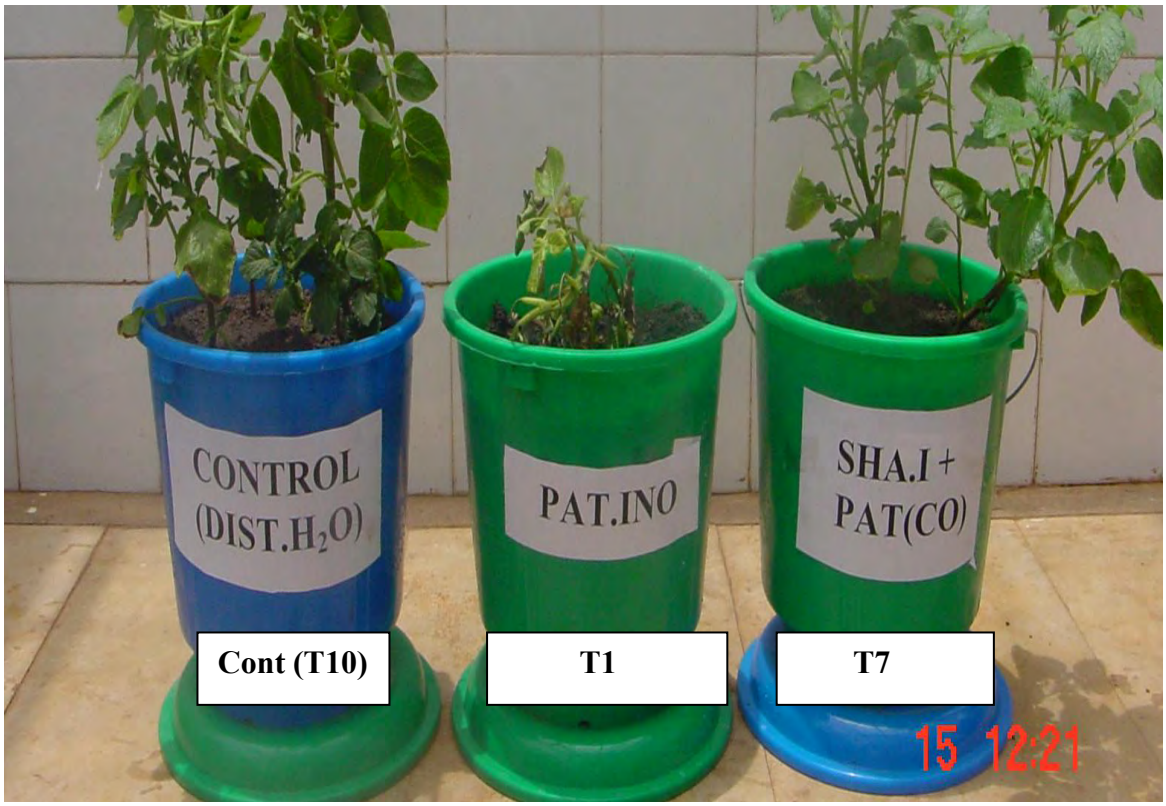

Figure 4. Greenhouse experiment showing difference between T1, T7 and T10

Table.7 Effect of bacterization with isolates of *P.fluorescens* on plant height and biomass

Treatment	Height▪ (Cm)	Dry weight▪ (gm)
T1. Rs262-b ino.	$20.83^{c} \pm 2.1$	$1.9^{c} \pm 1.51$
T2. Pfw1	$32.13^{a} \pm 1.1$	$11.97^{a} \pm 2.52$
T3. Pfs2	$32.87^{a} \pm 0.76$	$11.83^{a} \pm 1.19$
T4. Pfwt3	$32.83^{a} \pm 1.1$	$11.7^{a} \pm 2.41$
T5. Pfir	$32.53^{a} \pm 0.92$	$11.53^{a} \pm 0.95$
T6. Pfw1+ Rs262-b	$27.9^{b} \pm 1.8$	$6.77^{b} \pm 1.42$
T7. Pfs2+ Rs262-b	$26.77^{b} \pm 1.3$	$6.76^{b} \pm 1.2$
T8. Pfwt3+ Rs262-b	$27.17^{b} \pm 1.5$	$6.67^{b} \pm 1.12$
T9. Pfri+ Rs262-b	$26.5^{b} \pm 0.87$	$6.63^{b} \pm 1.24$
T10. Control	$27.4^{b} \pm 3.9$	$6.63^{b} \pm 1.5$

▪=Numbers are mean and S.D of three replicates (two plant in each pot)

* Means followed by the same letter with in a column are not significantly different by Tukey's test at α =0.05%.

5. Discussion

5.1 Pathogencity and Hypersensitivity test

The result of the tobacco leaf infiltration test showed that, the pathogen induced chlorosis of the infiltrated tobacco leaf which is the characteristics of race 3 (Marin and El - Nashaar, 1993) but do not cause wilting of the plant, therefore, the isolate belong to race 3 strain of *P.solanacearum* and tobacco is not the host of the isolate. Similarly, the pathogen caused wilting of the potato plant with in 3 to 6 days after inoculation that showed the isolate Rs 262-b was a virulent and potato is the host plant.

5.2 Characterization of Isolates

A total of 50 isolates of fluorescent pseudomonas were enriched using kings B medium. Fluorescien production, levan formation and morphological characteristics presented by the isolates on the medium are similar to the result reported by (Palleroni, 1993). The result of oxidase, catalase, starch hydrolysis, gelatine liquefaction, growth temperature, salt tolerance and carbohydrate utilization test agreed with the characteristics described for the species *P.fluoresences* (Stainer *et al*., 1966; Bossis *et al*., 2000). Although the characteristics of *P.fluorescens* were similar with that of *P.putida* the isolates were further differentiated on the basis of gelatine liquefaction test where all isolates of *P.fluorescens* were positive and all isolates of *P.putida* were negative Pickett *et al*., (1991). Similarly, the result of siderophore detection test agreed with result reported by (Arnow, 1937;Manninen and Sandholm, 1994).

The utilization of different carbon sources by the isolates proves the metabolic and ecological diversity of *P.fluorescens* (Palleroni, 1993), which presuppose its success of survival in the new environment where it is applied or treated as a biocontrol agent.

5.3 *In vitro* Antibiosis

In vitro antibiosis of the isolates were tested against the target pathogen on KB medium to evaluate its antagonistic efficiency. Then based on the diameter of inhibition zone, three isolates were selected for the green house experiment. In this test the smallest and largest inhibition zone of 1.2 and 2.4 mm diameter were caused by Pfri (Indian isolates) and Pfw1 (Wolayta isolates), respectively. Pfw1 having 200% efficiency being the most efficient isolates followed by Pfs2 and Pfwt3 with 125% and 111% efficiency, respectively. Similarly,(Savithiry and Gnanamanickam, 1987) and (Anuratha and Gnanamanickam, 1990) obtained 2.5 to 4cm and 1.0 to 2.8cm inhibition zones of diameter by *P.fluorescens* against *R .solani* and *R .solanacearum* respectively, on KB agar medium. Therefore, this three isolates along with the Indian reference strain were used as a candidate antagonist for the greenhouse experiment.

5.4 Green House Experiment

The green house experiment result suggested that the severity of bacterial wilt caused by *R .solanacearum* on potato plant could be reduced by bacterization of potato with selected isolates of fluorescent pseudomonas. This was supported by the greenhouse data for significant reduction of bacterial wilt in bacterized plant, Table 7.

The result of percent survival of potato tuber treated with selected isolates increased the survival of potato plant as compared to T1. Such increases in mean survival were 59.83%. This suggests a higher level of protection of potato from bacterial wilt and efficient isolates can be used in biological control of the disease. The statistical analysis of wilt suppression showed that there was no significant difference with in treatment (T2 to T6) but they differ significantly from T1. Similarly, Aspiras and Cruz (1985); Anuratha and Gnanamanikam (1990) and Gamliel and Katan (1993) reported that utilization of antagonistic rhizosphere bacteria such as *Bacillus spp*.; *P.fluorescens and P.putida* significantly increased the survival rate of potato, tomato, eggplant and cotton by 60-90%, 90% and 84-90% respectively against bacterial and fusarium wilt disease.

Potato tubers planted in pathogen-infested soil (T1) were severely damaged by the pathogen with the least biomass, plant height and percent survival than the rest of all treatment. However, bacterization of tuber with selected efficient isolates (T6-T9) significantly increased the plant height and dry weight by 76.89% and 28.44%, respectively as compared to T1. This result agreed with (Sivamari and Gnanamanickam, 1988) where they found an increased plant height and biomass of banana seedling by 62.17% and 61.54%, respectively due to bacterization with the suspension of *P .fluorescens* prior to planting in *F.oxysporium f.sp cubense* infested soil.

This is due to the fast growth rate of fluorescent pseudomonas relative to the pathogen followed by their aggressive root colonization nature that result in displacement of the pathogen (Suslow, 1982). In addition, the high substrate competitive and wide metabolic capability of fluorescent pseudomonas is also very important in this process. That is, it's ability to pre-emptively exclude the pathogen from the rhizosphere of potato plant was related to the nutritional similarity (high niche overlap) between the pathogen and the fluorescent pseudomonas also play a vital role in the wilt suppression and growth promotion of potato plant (Hoitink and Beohm, 1999).

Furthermore, all isolates used in the greenhouse experiment were found to produce siderophore which scavenge Fe (III) from the rhizosphere environment thereby starving the pathogen that give competitive advantage to the fluorescent pseudomonas, and induced systemic resistance which switch on the battery of all defense mechanism of the plant (Chen *et al*., 2000).

Potato tuber planted after bacterizatiion (T2-T5) only with the selected antagonistic isolates showed the most significant growth enhancement (Plant height and dry weight) when compared to potato plant in other treatments. This could be explained on the basis of the possibility of production of growth stimulating substance (hormone), nutrient solubilzation nature of the isolates. This suggests that the isolates may be the member of plant growth promoting rhizobacteria (Glick, 1995).

This result could also be interpreted in terms of interaction and specificity between fluorescent pseudomonas and potato plant. The isolates (Pfw1, Pfs2, Pfwt3) used in this study were well adapted to utilize exudates from its original host, potato, as it was isolated from potato rhizosphere. This has been substantiated by the close interaction between specific plant and the rhizosphere microorganisms (Whips, 2001). Such interaction would also enhance the plant protection and growth promotion rendered by this isolates.

Therefore, the evidence presented here is suggestive of the possibility of biological control for bacterial wilt of potato. In this particular experiment the isolates (*Pfw1, Pfs2, Pfwt3*) were capable of suppressing the pathogen both under laboratory and greenhouse conditions. Therefore, this data should be verified through extensive field test with better understanding of the physical and chemical factors that affect the ecology of the antagonist to increase the protection rendered by the isolates.

6. CONCLUSION AND RECOMMENDATION

Exploitation of the interaction between rhizosphere microorganisms is attracting a lot of interest in the biological control of soil borne phytopathogen.

In this experiment, 50 isolates of fluorescent pseudomonas were isolated and characterized. Then based on the result of *In vitro* inhibition test only three isolates (Pfw 1, Pfs 2, Pfwt 3 and one Indian strain) that inhibit the growth of the pathogen were selected and used in the green house experiment. The result showed that the selected antagonistic isolates managed to suppress or control the disease caused by the pathogen, *R. solanacearum* effectively. In addition they promoted the growth of the potato plant indicating the bacteria may be member of the plant growth promoting rhiozobacteria. Hence, *P .fluorescens* can be taken as good candidate of antagonistic bacteria to be used in the biological control of strategy of the economically important pathogen *R. solanacearum* which cause significant yield loss in the country.

Such kind of disease control is complicated because of the many variable factors such as host, strains of the pathogen, antagonistic bacteria and environment. So the impact of each factor must be further studied in the field so that the beneficial bacteria can control bacterial wilt effectively.

Though, plant protection rendered this way is very effective, the data showed that it is difficult to control bacterial wilt completely. Therefore, combining different

methods such as resistant variety and biocontrol together can give better protection than a single method. Moreover, addition or use of specific substrate that enhance selective growth and multiplication of the antagonist, use of multiple microbial inoculant rather than a single species alone, genetic manipulation of the desired isolate (the promising one), improving delivery of the formulation of the biocontrol agent etc, can be considered as untapped potential of *P.fluorescens* in the biological control of potato bacterial wilt in the country.

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APPENDIX

Appendix 1. Location and altitude of the study areas from which some representative isolates of *P.fluorescens* were recovered.

Name of Isolates	**Location**	**Altitude (m.a.s.l)**
*Pfa*1, *Pfa2, Pfa3, Pfa4, Pfa5, Pfa6, Pfa* 7,*Pfa* 8.	Ambo	2100-2250
*Pfb*1, *Pfb2, Pfb3, Pfb4, Pfb* 5, *Pfb 6, Pfb 7*	Bako	1650-1800
*Pfs*1, *Pfs*2, *Pfs*3, *Pfs*4, *Pfs5*, *Pfs*6, *Pfs*7, *Pfs*8, *Pfs9*, *Pfs*10	Shashamane	1800-2250
*Pfwt*1, *Pfwt2*, *Pfwt*3, *Pfwt*4, *Pfwt*5, *Pfwt*6, *Pfwt*7, *Pfwt*8, *Pfwt*9, *Pfwt*10	Wondogenet	1650-1750
*Pfw*1, *Pfw*2, *Pfw*3, *Pfw*4, *Pfw5*, *Pfw*6, *Pfw7*, *Pfw*8, *Pfw9*, *Pfw*10, *Pfw*11, *Pfw12*, *Pfw13, Pfw14, Pfw15*	Wolayta	----------

Appendix 2. Climatic Data of Ambo Plant Protection Research Center

Month	Rainfall (mm)	Temperature (^{O}C)		Humidity (%)
		Min.	Max.	
January	84.3	10.0	27.0	51.0
February	112.3	12.0	28.0	44.0
March	41.6	13.3	27.3	53.0
April	150.8	13.4	26.8	54.0
May	2.0	13.0	29.2	39.0
June	204.5	11.5	26.0	73.2
July	276.5	11.5	23.2	83.6
August	128.2	11.2	23.8	85.2
September	64.5	10.1	26.4	76.8
October	118.7	10.7	26.1	75.1
November	42.6	10.7	26.0	66.5
December	7.8	9.9	24.8	48.0
Total	1233.8	137.3	314.60	749.40
Mean	102.82	11.44	26.22	62.45

Appendix 3. Climatic Data of Bako Research Center

Month	Rainfall (mm)	Temperature (^{O}C)		Humidity (%)
		Min.	Max.	
January	4.0	13.4	30.4	48.0
February	34.3	15.4	32.6	48.0
March	51.7	15.3	31.1	51.0
April	59.1	16.4	31.1	50.0
May	5.7	16.8	32.9	43.0
June	265.1	14.8	26.2	68.0
July	420.6	15.6	23.3	73.1
August	434.4	15.4	23.3	73.0
September	69.9	15.0	24.0	67.0
October	21.5	13.9	26.9	57.0
November	1.2	12.7	30.0	55.0
December	27.6	11.3	30.5	51.0
Total	1395	176.0	342.3	684.1
Mean	116.26	14.67	28.53	57.01

Appendix: 4 ANOVA showing dry weight, plant height and percent survival of potato tuber treated with *P.fluorescens*

	Source	Sum of square	df	Mean square	F-value	Sig.
St. d wt	Between groups	305.379	9	33.931	13.445	< 0.001
	With in groups	50.473	20	2.524		
	Total	355.852	29			
Sh. Pt. Ht.	Between groups	408.212	9	45.357	26.535	< 0.001
	With in groups	34.187	20	1.709		
	Total	442.399	29			
% surv.	Between groups	6035.513	5	1207.103	738.359	< 0.001
	With in groups	19.618	12	1.635		
	Total	6055.131	17			